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(54) POROUS INORGANIC SUPPORT MATERIALS

(71) We, CORNING GLASS WORKS, a corporation organised under the laws of the State of New York, United States of America, of Corning, New York, N.Y. 14830, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to porous inorganic support materials, more specifically to improved porous inorganic support materials and to the use thereof. In particular, these materials are useful as carriers for, e.g., enzymes, antigens and antibodies.

High specific surface area inorganic materials consisting of at least one metal oxide have long been used as catalysts or support materials for catalysts. Typically, the inorganic materials are highly porous to provide specific surface areas as high as, or higher than, 100 M^2/g. Examples of such high specific surface area inorganic supports include various dried xerogels and porous glass bodies of the type described, for example, in United States Patent Nos. 3,549,524 and 3,485,687.

Inorganic support materials offer many advantages over high specific surface area organic supports. Among the advantages of the inorganic materials are their rigidity, stability, and non-swellability. Furthermore, inorganic materials are generally not subject to microbial attack, may readily be sterilized and are easier to handle, store, and use. Also, such inorganic materials are available in porous form, having very high specific surface areas. In many cases, porous inorganic materials are even less expensive to prepare than organic supports of comparable surface area.

Unfortunately, however, the utility of porous inorganic support materials as carriers for various catalytically active organic substances is limited because of difficulties and/or costs encountered in securely bonding the organic substances to the surface of the inorganic materials. For example, it has been shown in United States Patent No. 3,556,945, that enzymes may be adsorbed to the surface of high specific surface area porous glass. However, the adsorption bonds are relatively weak and are pH dependent, thus limiting the applications for this mode of attachment. Furthermore, since adsorption is a non-specific mode of attachment, the so-called "active site" of enzyme molecule may take part in the adsorptive bonding, thus limiting the catalytic activity of the resulting enzyme composite. Because of these difficulties, attempts have been made to find better and more specific methods of bonding enzymes to inorganic support materials.

Recently, in United States Patent No. 3,519,538, it was disclosed that silane coupling agents could be used to couple enzymes to inorganic materials chemically. The preferred silane coupling agents are molecules having two reactive portions—one which may preferentially combine with inorganic surfaces and an organo-functional portion, which may react with, or be tailor-made to react with, organic substances. Thus, the silane coupling agents may be used as a link to chemically couple organic substances to inorganic materials with covalent chemical bonds. Furthermore, since the organo-functional portions of the silanes may be tailor-made to react with specific groups on an enzyme, the enzyme may be coupled through groups which are not essential to the enzyme's activity. It has been further disclosed that silane coupling

agents may also be used to chemically couple antigens and antibodies, (United States Patent No. 3,652,761), and chelating agents, (United States Patent No. 3,886,080). Modified methods of using silane coupling agents to chemically couple enzymes may be found in United States Patent Nos. 3,669,841 and 3,715,278.

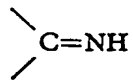
Although the above disclosures demonstrate that various organic substances may be successfully coupled to inorganic support materials, the steps involved are, in many instances, complicated, time consuming and costly. For example, several distinct steps are required when silane coupling agents are used. The inorganic carrier is commonly pre-treated for coupling of the silane. Once the silane is attached to the carrier, the organo-functional portion of the silane is often modified for final coupling of the enzyme. In many cases, e.g. where the organo-functional portion of the silane is diazotized, coupling of the enzyme must be accomplished shortly after the modification step, thus limiting storage of the treated carrier for a later, more convenient enzyme attachment. Furthermore, it is known that silanes may polymerize on the surface of the support materials, thus limiting control of the distance between the carrier and the substance linked through the silane.

Some of the above problems associated with the use of silane coupling agents are avoided or at least minimized by known methods for chemically coupling enzymes to organic carriers. Examples of various ways to chemically couple enzymes to organic carriers, e.g. cellulose derivatives, may be found in United States Patent No. 3,278,392. Furthermore, methods for chemically coupling enzymes to water-insoluble polymers using cyanogen halides are disclosed in United States Patent No. 3,645,852.

Although the above methods for immobilizing enzymes are relatively simple, they rely on the use of organic materials as supports. Hence, the advantages of high specific surface area porous inorganic carriers are not utilized. The only disclosure of which we are aware describing enzymes bonded chemically to inorganic materials without the use of silanes may be found in U.K. Patent No. 1,363,526. In that disclosure, however, the carriers, e.g. clay materials, lack the controlled porosity and hence do not exhibit the controllable high specific surface areas associated with high specific surface area porous materials, e.g. porous glass.

Quite surprisingly, a relatively simple procedure for chemically coupling various organic materials to porous high specific surface area inorganic supports has now been found. A critical feature of the present invention is the utilization of a novel support material.

The present invention provides a porous, substantially water-insoluble inorganic support material having a specific surface area of at least 5 m^2/g consisting of at least one metal oxide and having surface imino groups, i.e. groups corresponding to the formula:



The present invention also provides a method of making such materials having surface imino groups which may couple chemically with organic substances, which method comprises the step of reacting a porous, substantially water-insoluble inorganic material having a specific surface area of at least $5m^2/g$ consisting of at least one metal oxide and having a surface hydroxyl or oxide groups with an aqueous solution of cyanogen bromide.

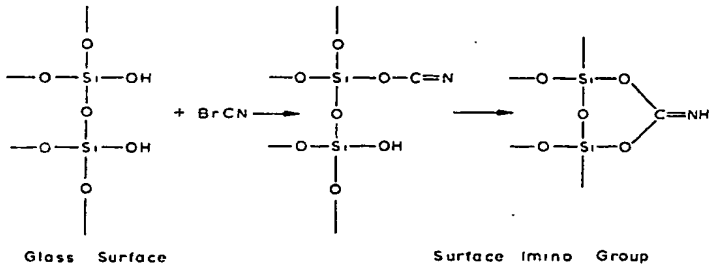
The support materials according to the present invention comprise high specific surface area porous inorganic bodies consisting of at least one metal oxide and having surface imino groups. The support materials are prepared by reacting high specific surface area, substantially water-insoluble, porous, inorganic bodies having surface hydroxyl or oxide groups with a solution of cyanogen halide, e.g. cyanogen bromide. The surface then becomes activated with surface imino groups which may then be reacted directly with the organic material to be attached or with an intermediate compound to which the organic material may subsequently be attached. Preferably, the porous inorganic support materials having surface imino groups which have a specific area of at least $5m^2/g$ consist of one or more metal oxides found in materials selected from porous glass particles, porous alumina and porous titania bodies, each having closely controlled average pore diameters.

The main requirements for the inorganic carries used for the preparation of the carriers according to the present invention are that they consist of at least one metal oxide, are substantially water-insoluble, porous and have a high specific surface area to permit high loading of the organic materials and have surface hydroxyl or oxide

groups capable of reacting with a cyanogen halide solution to yield surface imino groups. The specific surface area must be at least $5m^2/g$, preferably at least $50m^2/g$. Such specific surface areas may be found in various inorganic particles, e.g., dried xerogels and porous glass particles and beads. Preferably, porous inorganic particles of from 20 to 80 mesh (US Standard Sieve) are used. The ideal average pore size depends on the type of organic material which is ultimately coupled to the inorganic carrier. For example, when high molecular weight proteinaceous substances, e.g. enzymes, are to be chemically coupled to the porous carriers, the average pore diameter should be from 200 to 1,000 A, depending, e.g., on the size of the protein, the intended use of the protein and the intended use of the resulting composite. It should be noted that one of the very important features of the carriers is that they are porous and have large specific surface areas for maximum loading of organic materials. Preferably, the porous carriers have a very closely controlled average pore diameter within the above range, e.g. $\pm 10\%$.

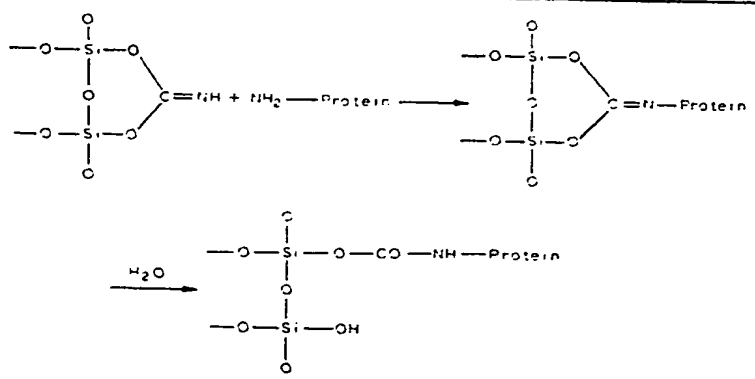
Porous glass particles which are especially useful as starting materials include, e.g., "Corning Code GZO-3900", porous glass particles having an average pore diameter of 550 Å $\pm 10\%$ and a typical specific surface area of approximately 70 M^2/g and also "Corning Code MZO-3900", zirconia-coated porous glass particles having an average pore diameter of 550 Å $\pm 10\%$ and a typical specific surface area of approximately 82 M^2/g.

The surface hydroxyl or oxide groups are needed on such particles for reaction with the cyanogen halide solution to yield the surface imino groups. The mechanism of the reaction with porous glass is thought to occur in the following manner where, for example, cyanogen bromide is thought to react with the surface hydroxyl groups of porous glass as follows:



Once the surface imino groups are formed, the surface-activated carriers are useful in numerous applications where it is desirable to chemically couple organic substances to a high specific surface area inorganic support. After the surface imino groups have been formed on the treated carriers, the carrier may be used to immobilize any organic material having a chemical group which will react with the surface imino group. Alternatively, intermediate compounds having sites which will react with imino groups may be used to form links of varying lengths between the inorganic carrier and the organic substance to be immobilized. For example, a diaminoalkane or a diaminobenzene may be reacted with the surface imino group to yield surface alkylamine or arylamine groups. The remaining amine group may be used to chemically couple various organic substances having groups which may react with an amine group. Alternatively, the remaining amine of, for example, an arylamine, may be chemically modified, e.g. diazotized, to be reactive with a wide variety of other groups which may be available on the organic substance to be immobilized.

In the more direct approach, however, the organic material to be immobilized reacts directly with the surface imino groups. For example, a protein, e.g. an enzyme, having amino groups which are not essential for enzyme activity, e.g. not a part of the enzyme's active site, is thought to react with porous glass having surface imino groups according to the following scheme:



It is thought that numerous such bonds are actually involved in the binding of a single such enzyme.

In the Examples below various enzymes were covalently coupled to samples of porous glass, zirconia-coated porous glass, porous titania, and porous alumina bodies which had been treated with cyanogen bromide to yield surface imino groups. After the enzymes were coupled to the surface-activated carriers, the composites were repeatedly washed in urea solutions to remove any adsorbed enzymes and the enzyme-carrier composites were then assayed for enzymatic activity. Enzymatic activities were found indicating chemical coupling of the enzymes.

Since the surface-treated inorganic carriers may be used to chemically couple a wide variety of organic materials which could not be coupled directly to inorganic carriers, it should be pointed out that the Examples below are given merely to demonstrate the utility of porous inorganic carriers having surface imino groups. Thus, for example, the treated carriers according to the present invention may be used to chemically couple other proteins, e.g. antibodies, proteinaceous antigens and other macromolecules having available functional groups. Chelating agents and other organic materials may be chemically coupled to the treated carriers in a manner somewhat analogous to the techniques disclosed above using silane coupling agents. Since the surface imino groups may readily be modified to other reactive groups, it is clear that a wide variety of reactive surfaces may be tailor-made from the surface imino groups of the treated carriers. Thus, where it is necessary to protect various reactive sites on a given organic substance, e.g. active sites, the treated carriers may be modified to react preferentially, or bond, with groups on the organic substance which are not essential for the activity or function of the substance.

In the preparation of the carriers according to the present invention, high specific surface area porous inorganic support materials are cleaned and dried, if necessary, to provide surfaces having reactive hydroxyl or oxide groups. As noted above, the inorganic support materials include a wide variety of materials, both amorphous and crystalline. Typically, the inorganic bodies initially consist of at least one metal oxide or combinatons of metal oxides, e.g. glass, which bodies are pre-treated, if necessary, to ensure a clean surface, e.g. by washing with weak acid followed by heating at slightly over 100°C.

Then, the inorganic bodies are mixed with an aqueous solution of a cyanogen halide, preferably cyanogen bromide, generally at a pH of from 9 to 12 and a temperature of from 0 to 25°C, for a period of time sufficient to form surface imino groups on at least the major portion of the carrier surface. The reaction of the carrier with the cyanogen halide solution is preferably over a period of at least five minutes, more preferably for approximately one hour. The pH of the reaction may be maintained using NaOH. The optimum concentration of the cyanogen halide solution will depend on the amount of porous carrier and the total surface area. As a practical matter, the treating solution should contain from 0.2 to 1 g of cyanogen halide per gram of dry inorganic material to be treated, (e.g. 20% to 100%, by dry weight).

After the surface imino groups have been formed, the treated carrier is removed from the reaction solution and thoroughly washed with distilled water or a bicarbonate solution at a pH of from 5 to 8.5.

At this point the treated carrier is ready for surface modification, by the addition of appropriate reactive groups, or for immediate use to chemically couple any organic substance having groups reactive with the imino groups, e.g. having amino groups. When proteins, e.g. enzymes, are chemically coupled to the surface imino

groups of the carrier, the coupling procedure is preferably carried out at a pH of from 7 to 9 and at a temperature of from 0 to 25°C. The amount of protein is chosen to maximize the amount which may be chemically coupled to the surface of a treated carrier having a given surface area. Preferably a thin aqueous slurry of protein solution is reacted with the surface-treated carriers.

The following Examples illustrate the invention:—

Example I

Surface-Activated Porous Glass

Surface imino groups were formed on high specific surface area porous glass as follows: To 20 g of porous "96% silica" glass (Corning Code GZO-3900) particles having an average pore diameter of 550 Å and particle size of from 20 to 80 mesh, United States Standard Sieve, (from 177 to 840 microns), was added 50 ml of an aqueous solution containing 5.0 grams cyanogen bromide at a pH of 11.0 and at a temperature of 0°C. The glass particles remained in contact with the solution for one hour and the pH was maintained by the dropwise addition of NaOH solution. The porous glass particles were then removed from the solution and washed with cold distilled water. The surface-treated porous glass particles were then ready for bonding to enzymes.

The surface-activated porous glass particles were added to an aqueous solution containing 2 grams of crystalline trypsin in enough distilled water to make a thin slurry. The pH was maintained at 8.5 and the mixture was stirred and allowed to react for two hours. The final product (immobilized trypsin) was then washed with distilled water and soaked in 6 M urea solution for one hour (to remove any adsorbed enzyme) before assay. A control sample was also prepared with similar porous glass particles which had not been surface-treated with cyanogen bromide prior to contact with the enzyme solution. The enzyme trypsin was used for both the surface-treated porous glass and the control porous glass since earlier work had indicated that trypsin tends to inactivate in time when adsorbed to porous glass. Thus, after assay of the treated carrier composite and the control, any activity noted for both samples would not be fully attributable to any adsorbed enzyme remaining even after the urea soakings.

The assay method for both samples was the standard TCA precipitate method for assaying the activity of trypsin with 1% casein as substrate at pH 7.0. Trypsin activity is expressed in standard units. For both samples, the assays were preformed with 5 mg composite samples (dry weight). After the initial assays, both samples were re-soaked in 6 M urea solutions for one hour, followed by further assays, soakings, and re-assays according to the Table below. The Table shows the activities of the respective samples after each successive urea soaking. The activity found at each assay is expressed in mg. trypsin per gram carrier, on a dry weight basis.

TABLE

Trypsin Activity After 1 Hour Soaks in 6 M Urea

Soak No.	Observed Activity (mg/g Carrier)	
	Control Carrier	Treated Carrier
1	1.0	10.8
2	—	7.4
3	1.0	6.4
4	0.6	—
5	<0.2	7.4
6	—	6.4
7	—	6.0

From the above Table, it may be concluded that covalent bonding of the trypsin to the treated carrier did occur, thus confirming the utility of the surface-treated carriers.

Example II

Surface-Activated Zirconia-Coated Porous Glass

As in Example I, a similar cyanogen bromide solution was used to activate the surface of similar porous glass particles (e.g. average pore diameter of 550 Å and mesh size of from 20 to 80), which had been thinly coated with a surface layer of zirconia in accordance with the disclosure in United States Patent No. 3,783,101. "Zirconia-coated porous glass" refers to porous glass as prepared according to that

disclosure. The glass used in this Example is known as "Corning Code MZO-3900" zirconia-coated porous glass. Zirconia coatings on the surface of porous glass have been found effective in enhancing the alkaline durability of the glass particles. The zirconia-coated glass particles were surface-treated with the cyanogen bromide solution as in Example I. After the surface-treatment, 5 g samples of the treated zirconia-coated glass particles and control zirconia-coated glass particles were exposed to a trypsin solution under the reaction conditions as described in Example I. After a total of seven one-hour washes with 6 M urea solutions, the composite consisting of trypsin bonded to the carrier which had been surface-treated with the cyanogen bromide solution was assayed and found to have a trypsin activity of 2.8 mg trypsin per gram zirconia-coated carrier. The control carrier, after similar urea washes, was assayed and found to have a trypsin activity of only 0.4 mg trypsin per gram carrier.

Example III
Surface-Activated Porous Glass

Example I was repeated using similar materials and conditions. After three one-hour washings in the 6 M urea solution, assays were taken of the control and the composite consisting of trypsin bonded to the porous glass carrier that had been treated with the cyanogen bromide solution. The activity of the control composite was found to be 1.0 mg trypsin activity per gram carrier. The activity of the composite using the treated carrier was found to be 6.4 mg trypsin activity per gram carrier, thus essentially confirming the results of Example I.

Example IV
Surface-Activated Porous Glass

The porous glass particles were activated as described in Example I and then contacted with a glucose isomerase solution. An unactivated control carrier was also exposed to a similar glucose isomerase solution under similar conditions. Both composites were washed and then assayed under standard conditions for determining glucose isomerase activity wherein one unit of activity represents the production of 1 μ mole/min. of fructose at 60°C., pH 6.85. The composite consisting of glucose isomerase bonded to the carrier which had been treated with the cyanogen bromide solution was found to have 800 units per gram of carrier. The control composite was found to have 50 units activity per gram carrier.

Example V
Surface-Activated Porous Alumina

10 grams porous alumina bodies of from 20 to 40 mesh, United States Standard Sieve, having an average pore diameter of 400 Å were treated with a cyanogen bromide solution as in Example I. The porous bodies were made in accordance with the methods disclosed in United States Patent No. 3,892,580. The surface-treated carrier was then exposed to similar glucose isomerase solutions (as in Example IV) and, after standard assay, the composite showed an activity of 280 units per gram alumina.

Example VI
Surface-Activated Porous Titania

10 grams porous titania bodies of from 20 to 60 mesh, United States Standard Sieve, also made in accordance with the teachings of the above document, having an average pore diameter of 185 Å were surface-treated with a cyanogen bromide solution at pH 11.0 for 1 hour. The surface-treated titania bodies and an untreated control were then exposed to similar glucose isomerase solutions for from 1 to 2 hours. After assay, the control was found to have 680 units of glucose isomerase activity per gram carrier. The treated carrier had an activity of 380 units per gram carrier, probably indicating a high degree of adsorption of the enzyme without significant activity loss, even after washing with distilled water; (c.f. next Example).

Example VII
Surface-Activated Porous Titania
(Half-Life Studies)

10 grams porous titania bodies as described in Example VI were again exposed to a similar cyanogen bromide solution for 1 hour at pH 11.0 and the surface-treated bodies and control bodies (untreated) were each exposed to the same amount of glucose isomerase in solution under similar conditions (for 2 hours, at a temperature of 0°C. and pH 8.5). 10 gram aliquots of the samples were washed with the urea solu-

tion and then placed in small plug flow columns. A 50% glucose solution at pH 7.8 in 0.005 M $MgSO_4$ and 0.005 M Na_2SO_3 was continuously flowed into each column to determine the respective half-lives of the composites. The composites using the surface-activated carrier had an initial enzyme loading of 221 units per gram composite. The enzymatic half-life of the composite was found to be 15.3 days with a 95% upper confidence limit (UCL) of 31.7 days and a 95% lower confidence limit (LCL) of 10.3 days. The control composite was found to have an initial loading (adsorbed enzyme) of 525 units per gram composite, but three washings with the 6 M urea solution removed all enzyme activity.

Example VIII

Surface-Activated Zirconia-Coated Porous Glass

10 grams surface-treated zirconia-coated porous glass particles as described in Example II ("Corning Code MZO-3900" zirconia-coated porous glass) were exposed to a glucoamylase solution for 2 hours as was a non-treated control carrier. After standard assays for glucoamylase activity, the composite consisting of glucoamylase bonded to the treated carrier was found to have 792 units glucoamylase activity per gram carrier, where 1 unit represents the production of 1 μ mole/minute of glucose at 60°C., pH 4.0. The control sample (having the untreated carrier) was found to have 1859 units glucoamylase activity per gram carrier, again probably indicating a high degree of enzyme adsorption without significant loss of enzyme activity; (c.f. next Example).

Example IX

Surface-Activated Zirconia-Coated Porous Glass (Half-Life Studies)

10 gram aliquots of the samples of Example VIII (control and composite having the treated carrier) were placed in small flow-through columns of the type used in Example VII. A solution of enzyme-thinned cornstarch at 30% solids, pH 4.5 was continuously run through the columns at 55°C. and the half-lives of both samples were determined. The composite consisting of glucoamylase bonded to the carrier which had been treated with the cyanogen bromide solution was found to have a half-life of 25 days with a 95% UCL of 28.5 days and a 95% LCL of 21 days. The control sample (untreated carrier with adsorbed enzyme) was found to have a half-life of 4.9 days with a 95% UCL of 6.4 days and a 95% LCL of 4.0 days.

WHAT WE CLAIM IS:—

1. A porous, substantially water-insoluble inorganic support material having a specific surface area of at least 5 m^2/g consisting of at least one metal oxide and having surface imino groups.
2. A material as claimed in claim 1 in which the said specific surface area is at least 50 m^2/g.
3. A material as claimed in claim 1 or claim 2 in which the material having the surface imino groups comprises a porous glass particle.
4. A material as claimed in claim 3 in which the porous glass particle has an average pore diameter of from 200 to 1000 Å and a particle size of from 20 to 80 mesh, United States Standard Sieve.
5. A material as claimed in claim 1 or claim 2 in which the material having the surface imino groups comprises a zirconia-coated porous glass particle.
6. A material as claimed in claim 5 in which the zirconia-coated porous glass particle has an average pore diameter of from 200 to 1000 Å and a particle size of from 20 to 80 mesh, United States Standard Sieve.
7. A material as claimed in claim 1 or claim 2 in which the material having the surface imino groups comprises a porous alumina body.
8. A material as claimed in claim 1 or claim 2 in which the material having the surface imino groups comprises a porous titania body.
9. A material as claimed in claim 1 substantially as herein described.
10. A material as claimed in claim 1 substantially as herein described with reference to any one of the Examples.
11. A method of making an inorganic support material as claimed in claim 1, having surface imino groups which may couple chemically with organic substances, which method comprises the step of reacting a porous, substantially water-insoluble inorganic material having a specific surface area of at least 5 m^2/g consisting of at least one metal oxide and having surface hydroxyl or oxide groups with an aqueous solution of cyanogen bromide.

12. A method as claimed in claim 11 in which the reaction is carried out at a pH of from 9 to 12.

13. A method as claimed in claim 11 or claim 12 in which the reaction is carried out at a temperature of from 0 to 25°C.

14. A method as claimed in any of claims 11 to 13 in which the porous inorganic material has an average pore diameter of from 200 to 1000 A.

15. A method as claimed in any of claims 11 to 14 in which the cyanogen bromide solution contains from 0.2 to 1.0 g cyanogen bromide per gram of porous inorganic material.

16. A method as claimed in claim 11 substantially as herein described.

17. A method as claimed in claim 11 substantially as herein described with reference to any one of the Examples.

18. An inorganic support material prepared by a method as claimed in any of claims 11 to 17.

19. A composite material comprising an inorganic support material as claimed in any of claims 1 to 10 or 18 and at least one organic substance chemically coupled thereto.

20. A composite material as claimed in claim 19 in which at least one of the said organic substances is an enzyme.

21. A composite material as claimed in claim 19 or claim 20 substantially as herein described.

22. A method of producing a composite material as claimed in any of claims 19 to 21 substantially as herein described.

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